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Squeezing Axons Out of the Gray Matter: A Role for Slit and Semaphorin Proteins from Midline and Ventral Spinal Cord

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Summary

Commissural axons cross the nervous system midline and then turn to grow alongside it, neither recrossing nor projecting back into ventral regions. In *Drosophila*, the midline repellent Slit prevents recrossing: axons cross once because they are initially unresponsive to Slit, becoming responsive only upon crossing. We show that commissural axons in mammals similarly acquire responsiveness to a midline repellent activity upon crossing. Remarkably, they also become responsive to a repellent activity from ventral spinal cord, helping explain why they never reenter that region. Several Slit and Semaphorin proteins, expressed in midline and/or ventral tissues, mimic these repellent activities, and midline guidance defects are observed in mice lacking neuropilin-2, a Semaphorin receptor. Thus, Slit and Semaphorin repellents from midline and nonmidline tissues may help prevent crossing axons from reentering gray matter, squeezing them into surrounding fiber tracts.

Introduction

The midline of the central nervous system (CNS) is an important source of guidance information for developing axons navigating to their targets (reviewed in Colamarino and Tessier-Lavigne, 1995). In both vertebrates and invertebrates, axons are attracted to the midline in part by chemoattractants of the netrin family. Once there, different populations of axons take divergent trajectories, with some turning to remain ipsilaterally and others (the so-called commissural axons) crossing the midline to enable the transfer of information from one side of the body to the other. The divergent trajectories of ipsilaterally projecting axons and of commissural axons at the midline appear to be controlled by short-range guidance cues, both attractive and repulsive, that are expressed by midline cells and cells flanking the midline (Colamarino and Tessier-Lavigne, 1995).

An intriguing aspect of commissural axon behavior in all organisms is that these axons cross the midline only once, never recrossing under normal circumstances despite the fact that many of them subsequently grow alongside the midline for considerable distances. What appears surprising about this behavior is that the axons apparently find the midline to be a favorable environment for growth the first time they encounter it but not after they have crossed. In *Drosophila*, this apparent switch in preferences has been shown to be caused by the tight spatial regulation of expression of the Roundabout (Robo) protein, a transmembrane receptor for a repellent protein, Slit, made by midline cells (Kidd et al., 1998a, 1998b, 1999). Commissural axons express the Robo receptor on their surfaces, but the level of expression is kept low prior to midline crossing by the action of a negative regulator, the Commissureless (Comm) protein, enabling the axons to cross a first time (Kidd et al., 1998a, 1998b). After crossing, however, this repressive influence is somehow relieved so that commissural axons acquire high-level expression of Robo and, consequently, become highly responsive to the Slit repellent, explaining why they can no longer recross. This model is consistent with the results of extensive genetic analysis. For example, commissural axons that lack Robo function (in *robo* mutants) can cross the midline multiple times, whereas in *comm* mutants, commissural axons express high levels of Robo protein on their surfaces as soon as they are initiated and fail to cross the midline (Seeger et al., 1993; Kidd et al., 1998b). Thus, expression of Robo on an axonal surface is correlated with its inability to cross the midline.

These initial studies in *Drosophila* have left open a number of important questions. First, are the mechanisms regulating midline crossing phylogenetically conserved? Initial studies in vertebrates suggested that a variant mechanism might be at play. In chick embryos, spinal commissural axons express axonin-1/TAG-1, a receptor for the cell adhesion molecule NrCAM expressed by midline floor plate cells, and inhibition of axonin-1 or NrCAM function in vivo using function-blocking reagents results in a failure of midline crossing by large numbers of commissural axons, which instead turn ipsilaterally upon encountering the midline (Stoeckli and Landmesser, 1995). These studies and in vitro analysis of encounters of spinal commissural axon growth cones with isolated floor plate cells in vitro in the presence of the same function-blocking reagents (Stoeckli et al., 1997) suggested a model in which floor plate cells express an unknown midline repellent to which the axons are already responsive prior to midline crossing, but that the action of this repellent is masked by the action of midline NrCAM. The fact that TAG-1 expression on commissural axons is lost after midline crossing (at least in rodents) (Dodd 88) suggests that the inability of commissural axons to recross the midline after crossing might result from a loss of responsiveness to the positive factor NrCAM. Consistent with a loss of responsiveness to positive factors, commissural axons in the hindbrain were, in fact, shown to lose responsiveness to the attractive factor netrin-1 upon midline crossing (Shirasaki et al., 1988).

Thus, the evidence suggests that commissural axons in vertebrates are already responsive to a negative midline factor(s) prior to crossing and may be prevented

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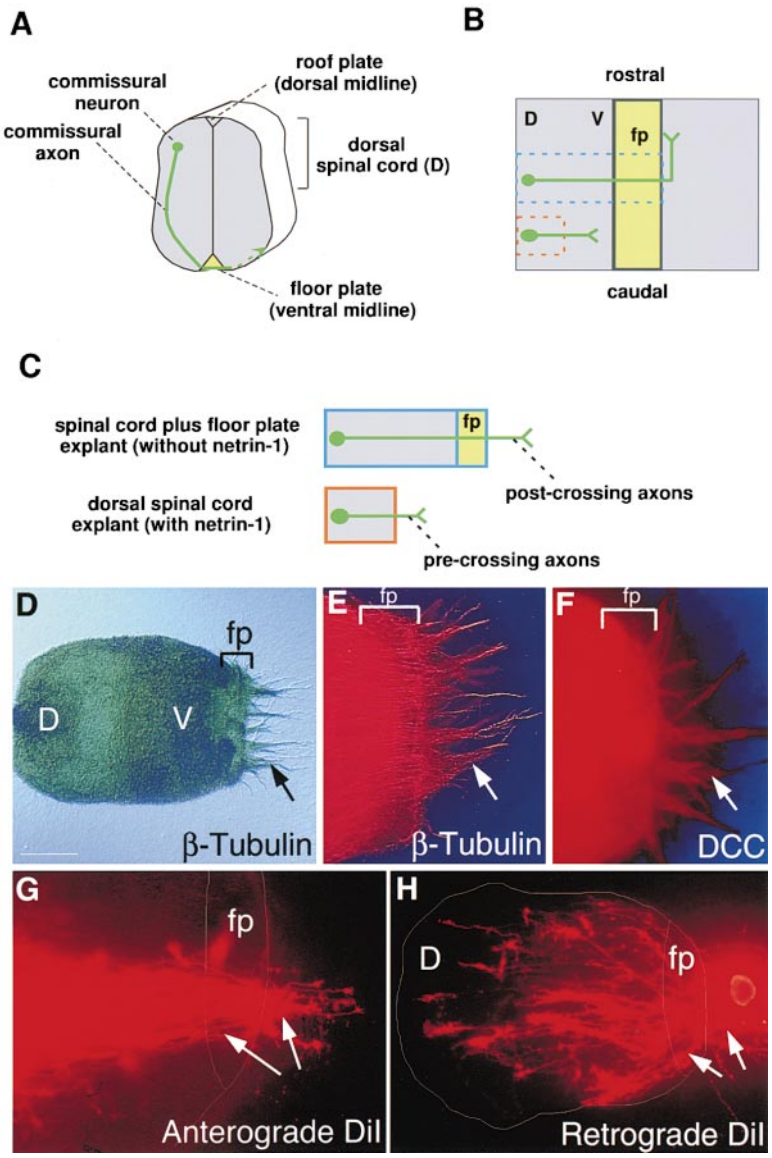


Figure 1. An In Vitro Explant Assay to Study the Responsiveness of Axons after Floor Plate Crossing

(A) Diagram illustrating the dorsoventral trajectory of commissural axons in the developing spinal cord. Commissural neuron cell bodies and axons are in green. The floor plate is in yellow.

(B) "Open book" view of the spinal cord showing the rostral turn of commissural axons after midline crossing. Blue dotted line indicates where a cut is made to generate the "spinal cord plus floor plate" explant. Orange dotted line indicates where a cut is made to generate the dorsal spinal cord explant.

(C) Diagram of the appearance of axons after explant culture, with post-crossing axons and pre-crossing axons growing into the collagen matrix.

(D–F) Post-crossing axons (emerging from a "spinal cord plus floor plate explant" after culture) are visualized with an anti-β-tubulin monoclonal antibody (D and E) or an anti-DCC antibody (F), and HRP-conjugated (D) or Cy3-conjugated (E and F) secondary antibodies.

(G) Many of the post-crossing axons that enter the collagen from "spinal cord plus floor plate" explants are labeled following implantation of a DiI crystal into the dorsal spinal cord, thus identifying them as commissual axons.

(H) Insertion of a DiI crystal next to the axons that have projected into the collagen gel from a "spinal cord plus floor plate explant" labels post-crossing axons emanating from cell bodies at several levels along the dorsoventral axis of the spinal cord, including the dorsal-most spinal cord.

Abbreviations: D, dorsal spinal cord; V, ventral spinal cord; fp, floor plate. Scale bar: (D) and (H), 200 μm; (E)–(G), 100 μm.

from recrossing the midline partly because they lose responsiveness to positive factors. Whether they also acquire responsiveness to additional negative factors at the midline, as in *Drosophila*, has not been determined. Three vertebrate homologs of Slit were recently identified and shown to function as repellents for various axonal classes, and to be expressed by midline floor plate cells. However, initial tests failed to demonstrate any effect of Slit proteins on spinal commissural axons (Brose et al., 1999; Li et al., 1999). Furthermore, in the hindbrain assay of Shiraski et al. (1998), commissural axons were shown to lose their attractive response to floor plate cells, but they also very clearly did not acquire a repulsive response to floor plate cells. Thus, the data are not just inconclusive; if anything, they might actually suggest that commissural axons do not acquire responsiveness to a diffusible midline repellent upon crossing.

Here we revisit this issue and show that the initial failure to show acquisition of a response to a repulsive floor plate activity resulted from idiosyncrasies of the assays used. Through the development of a novel in vitro assay that tests the behavior of post-crossing commissural axons in the spinal cord, we show that these axons do indeed acquire responsiveness to a repellent activity made by floor plate cells and that, surprisingly, this activity appears to be due not just to Slit proteins but also to repellents of the Semaphorin family.

A second issue not addressed so far in any organism is why commissural axons after crossing not only do not recross the midline but also, in many cases, do not reenter the ventral region of the nervous system (adjacent to the midline) through which they navigated to the midline but instead turn to grow alongside the midline. Here we use our novel in vitro assay to show that the axons also acquire responsiveness to a repellent activity made by ventral neural tissue, and again, we implicate Slit and Semaphorin proteins in mediating this effect.

Taken together, our results support a major extension of the *Drosophila* model, suggesting that at least in vertebrates, and perhaps in all organisms, commissural axons fail to recross the midline both because of loss

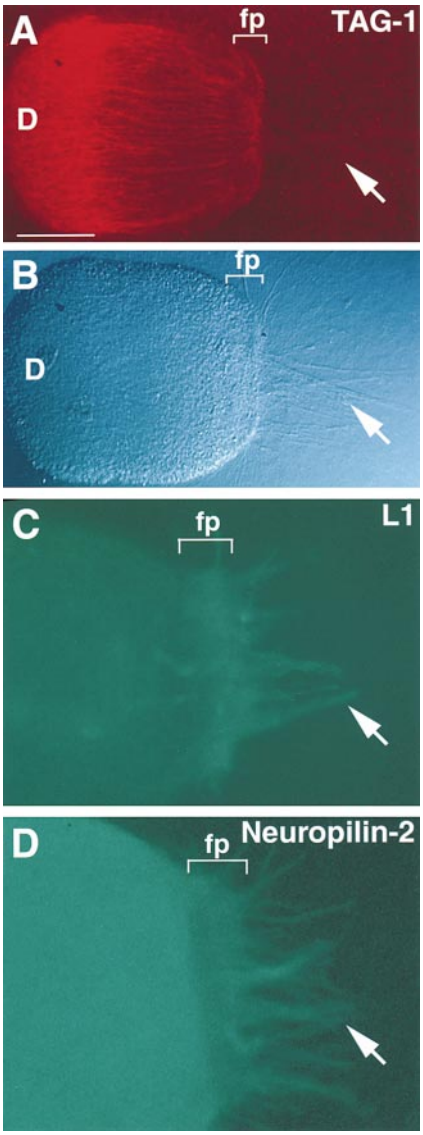


Figure 2. Post-Crossing Axons Emerging from "Spinal Cord Plus Floor Plate" Explants Have the Same Marker Expression Profile as Post-Crossing Commissural Axons In Vivo

(A and B) Post-crossing axons (white arrows) emerging from a "spinal cord plus floor plate" explant after crossing the floor plate (fp) can be visualized by phase contrast microscopy (B) but do not express TAG-1 (which labels commissural axons within the explant) (A). Note that the staining within the ventral spinal cord appears less intense than in the dorsal spinal cord; however, this does not reflect a real difference but rather the fact that the axons are in multiple different planes of focus in the ventral spinal cord, unlike the dorsal spinal cord. In contrast, within the collagen matrix, the post-crossing axons do not show any staining, whatever the plane of focus examined.

(C and D) Post-crossing axons (white arrows) emerging from other "spinal cord plus floor plate" explants express L1 (C) and neuropilin-2 (D) (the latter is also expressed by many cells within the explant).

Scale bar: (A) and (B), 200 μm; (C) and (D), 80 μm.

of responsiveness to positive midline factors and because of acquisition of responsiveness to negative midline factors and that they fail to reenter ventral neural tissue for the same reason.

Results

An In Vitro Assay to Assess the Behavior of Axons after Midline Crossing

To begin to study molecular cues that guide spinal commissural axons after they cross the midline floor plate, we developed a novel in vitro explant assay. In the rat, commissural axons are born in the dorsal spinal cord between embryonic days 11 and 13 (E11–E13) and extend axons that reach the floor plate about a day later, before crossing the midline and turning to project alongside the midline (Altman and Bayer, 1984; Dodd et al., 1988). Figure 1A illustrates the dorsal–ventral trajectory of commissural axons to the floor plate in the transverse plane, whereas Figure 1B diagrams the trajectory of these axons to and across the midline, as visualized in an "open book" preparation, in which the spinal cord is opened at the dorsal midline. In our assay, E13 spinal cords were prepared in this "open book" configuration and then cut as illustrated by the blue dotted lines in Figure 1B to give a hemisected spinal cord with floor plate attached. When these "spinal cord plus floor plate" explants were cultured in three-dimensional collagen gels for 16 hr, axons extended from the explants into the collagen mostly at right angles to the floor plate ("post-crossing" axons in Figure 1C), as seen by phase contrast microscopy (e.g., Figure 2B) and by immunohistochemistry using an anti-β-tubulin antibody (Figures 1D and 1E) and an anti-DCC antibody (Figure 1F). For comparison, dorsal spinal cord explants were dissected out as indicated in orange dotted lines in Figure 1B. In the presence of netrin-1 (but not its absence), pre-crossing axons grow into the collagen ("pre-crossing axons" in Figure 1C), as seen by phase contrast microscopy (e.g., Figures 3D–3F; Serafini et al., 1994).

To identify the location of the cell bodies of origin of the post-crossing axons, we performed anterograde and retrograde DiI tracing experiments. When a small DiI crystal was inserted in the dorsal-most portion of such an explant, anterogradely labeled axons, which are expected to be mostly or exclusively commissural axons at this stage (Altman and Bayer, 1984), were seen crossing the floor plate and entering the collagen gel (Figure 1G). In the converse type of experiment, a DiI crystal inserted next to axons that had entered the collagen gel was found to retrogradely label axons originating from cell bodies at various levels along the dorsoventral axis on the contralateral side of the floor plate (Figure 1H). Since the axons labeled in this way had crossed the floor plate, we assume that they correspond to commissural axons (which by definition are the crossing axons in vivo). The location of the cell bodies of these axons is consistent with this possibility, since commissural neuron cell bodies are located all along the dorsoventral axis (Altman and Bayer, 1984; Silos-Santiago and Snider, 1992; Liem et al., 1997). The expression of the surface markers DCC, TAG-1, and L1 (all members of the immunoglobulin superfamily), which was detected by immunohistochemistry using specific monoclonal antibodies, is also consistent with this possibility. DCC was detected on the axons both before and after midline crossing (Figure 1F), consistent with its expression on commissural axons in vivo (Keino-Masu et al., 1996). TAG-1, in contrast, was not expressed on the post-crossing portions of the axons and was detected only on pre-crossing axons (Figures 2A and 2B), consistent

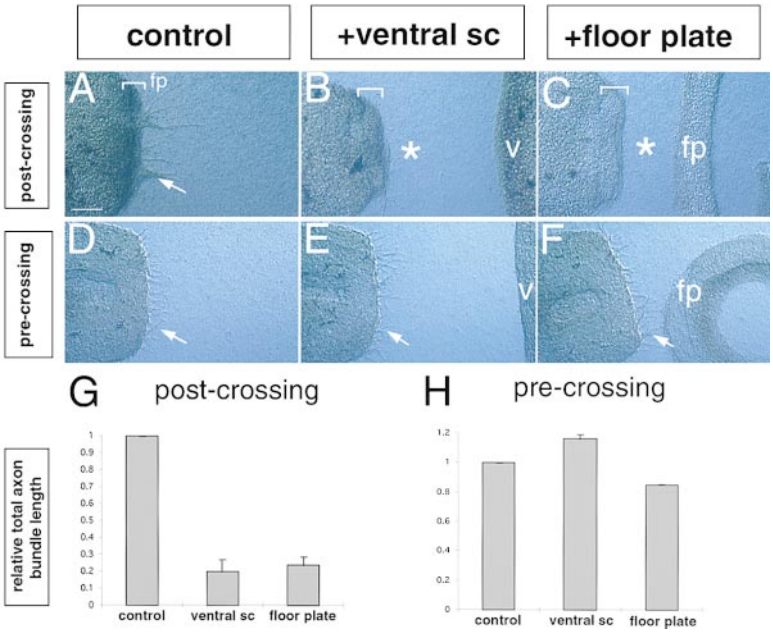


Figure 3. Inhibition of Post-Crossing Axon Growth into Collagen by Diffusible Activities from Ventral Spinal Cord and Floor Plate

(A–C) Post-crossing axons emerge from "spinal cord plus floor plate" explants cultured alone (A), but this outgrowth is suppressed by explants of ventral spinal cord (v) (B) or floor plate (fp) (C).

(D–F) In contrast, ventral spinal cord (E) and floor plate (F) explants do not suppress the outgrowth of axons emerging from dorsal spinal cord explants cultured with netrin-1.

(G and H) Quantification of total axon outgrowth seen in the presence of ventral spinal cord and floor plate explants, normalized to that observed in controls, for post-crossing axons (G) and for pre-crossing axons growing out of dorsal spinal cord explants in the presence of netrin-1 (H).

Scale bar: 200 μm.

with the fact that surface expression of TAG-1 is downregulated on commissural axons during midline crossing (Dodd et al., 1988). In control experiments, we examined pre-crossing axons that extended into collagen gels from E13 dorsal spinal cord explants in response to netrin-1 and that were found to express TAG-1 (data not shown), as previously described (Serafini et al., 1994). High-level L1 expression was observed on axons that had entered the collagen from the cut edge of the floor plate but not on axons still in the spinal cord explant (Figure 2C) or on pre-crossing axons that grew out in response to netrin-1 (data not shown), again consistent with the fact that commissural axons express L1 only after crossing, not before (Dodd et al., 1988). Finally, neuropilin-2 is expressed on commissural axons both before and after crossing (Chen et al., 1998; data not shown), and a neuropilin-2 antiserum labeled both the post-crossing fibers and the explant itself (Figure 2D).

The fact that the "post-crossing" axons in the in vitro assay cross the midline and express DCC and L1 but not TAG-1 is consistent with the possibility that they are commissural axons. In the absence of more specific markers to distinguish commissural axons from non-commissural axons, we cannot formally exclude that some axons that would not normally cross the floor plate do so in this in vitro assay, even if this seems unlikely. To reflect this residual uncertainty, we will continue to refer below to the axons that emerge from the floor plate as "post-crossing axons" rather than commissural axons.

The Ventral Spinal Cord and the Floor Plate Inhibit Post-Crossing but Not Pre-Crossing Axons

After commissural axons exit the floor plate, they enter ventral fiber tracts rather than recrossing the floor plate or reentering the ventral spinal cord. Since in *Drosophila* commissural axons that cross the midline become responsive to a midline repellent, Slit, we examined whether floor plate tissue can repel the post-crossing axons in our assay. Floor plate tissue inhibited the extension of post-crossing axons into collagen in our assay when placed at a distance (Figures 3C and 3G) but, as expected from previous studies (Tessier-Lavigne et al., 1988; Wang and Tessier-Lavigne, 1999), did not inhibit extension of uncrossed commissural axons projecting from dorsal spinal cord explants in response to netrin-1 (Figures 3F and 3H). Strikingly, we found that ventral spinal cord tissue also inhibited the outgrowth of post-crossing axons (Figures 3B and 3G) but not of pre-crossing axons (Figures 3E and 3H), demonstrating the existence in both floor plate and ventral spinal cord of a diffusible inhibitory activity (or activities) that suppresses the outgrowth of post-crossing axons.

The Class 3 Semaphorins and Slit Proteins Are Candidates for Mediating the Post-Crossing Axon Inhibitory Activity

During the period of their growth to and across the midline, commissural neurons express mRNA for the class 3 Semaphorin receptor neuropilin-2 (Chen et al., 1997), and commissural axons express neuropilin-2 protein (Figure 2D, and data not shown). They do not, however, appear to express neuropilin-1 (Chen et al., 1997; He and Tessier-Lavigne, 1997; Kolodkin et al., 1997). Neuropilin-2 is known to be required for mediating repulsive actions of the Semaphorins Sema3B, 3C, and 3F, whereas neuropilin-1 is known to be required for Sema3A function. In fact, Sema3B and Sema3F seem to require only neuropilin-2, not neuropilin-1, to mediate their effects, whereas Sema3C may require both neuropilin-1 and neuropilin-2 (Chen et al., 1998; de Castro et al., 1999). Although the expression patterns of several class 3 Semaphorins have been studied at various stages in the spinal cord (Luo et al., 1995; Püschel et al., 1995, 1996; Shepherd et al., 1997; Christensen et al., 1998), a systematic examination at the time of initial commissural axon growth has not been performed. We therefore examined the expression of the five known mammalian class 3 Semaphorin genes, *Sema3A*, *B*, *C*, *E*, and *F* (*Sema3D/collapsin-2* is a chick gene) (Semaphorin Nomenclature Committee, 1999). We examined these in the mouse because of the availability of probes for all these genes; gene expression patterns were examined

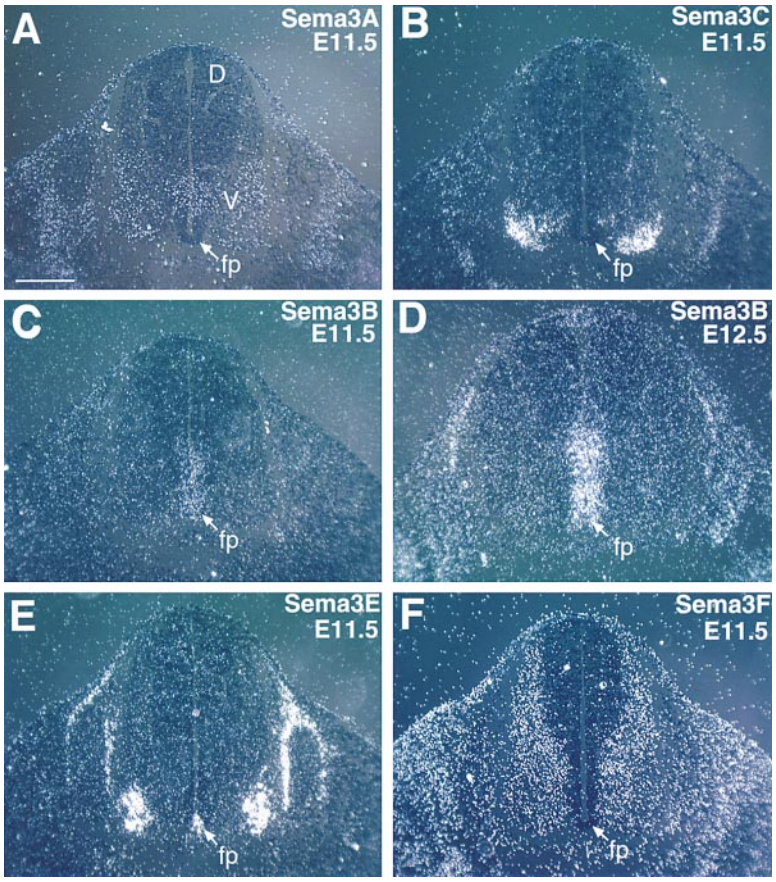


Figure 4. Expression Pattern of the Class 3 Semaphorins in the Spinal Cord

Expression of mRNAs for *Sema3A* (A), *Sema3B* (C and D), *Sema3C* (B), *Sema3E* (E), and *Sema3F* (F) visualized by in situ hybridization in transverse sections of the E11.5 mouse spinal cord (A–C, E, and F) or E12.5 mouse spinal cord (D).
Scale bar: 200μm.

at E11.5, which corresponds to E13 in the rat. As shown in Figure 4, all these genes are expressed in the spinal cord at this stage. *Sema3A* is expressed in the ventral horns and part of the ventral ventricular zone, in a pattern that presages its previously characterized expression pattern at later stages (Messersmith et al., 1995) but at lower levels (Figure 4A). *Sema3B* is found in the floor plate and ventral ventricular zone, increasing in intensity over time (Figures 4C and 4D). *Sema3C* and *Sema3E* are expressed in more restricted regions of the ventral horns than *Sema3A*, and in addition, *Sema3E* is expressed in the medial-most portion of the floor plate (Figures 4B and 4E). Finally, *Sema3F* is expressed very widely in the spinal cord, throughout the mantle zone but excluding the ventricular zone and floor plate (Figure 4F). Thus, based on expression of their mRNAs, Sema3B and 3E are candidates for contributing to the inhibitory actions of the floor plate, whereas Sema3A, 3C, 3E, and 3F are candidates for contributing to the repulsive actions of the ventral spinal cord. In a similar way, Slit-1, -2, and -3 are all candidates for contributing to the inhibitory actions of floor plate, and Slit-2 is a candidate for contributing to the inhibitory action of the ventral spinal cord, based on the expression pattern of their mRNAs (Brose et al., 1999; Li et al., 1999).

Slit-2 and Subset of Class 3 Semaphorins Can Inhibit Post-Crossing Axons

We tested the ability of class 3 Semaphorins and Slit-2 to mimic the inhibitory action of tissues on post-crossing axons by confronting those axons with aggregates of COS cells secreting these factors in the in vitro explant assay of Figures 1, 2, and 3. Cells secreting Sema3B (Figure 5E) or Sema3F (Figure 5H) strongly inhibited the outgrowth of the crossed axons, as did cells secreting Slit-2 (Figure 5C). Cells secreting Sema3A, 3C, or 3E had no effect in this assay, nor did cells secreting netrin-1 (Figures 5B, 5D, 5F, and 5G). These results are quantified in Figure 5Q. In contrast, when cells secreting these factors were presented to commissural axons growing out of dorsal spinal cord explants in response to netrin-1, none of the factors had an inhibitory effect on the axons (Figures 5I–5P). The results are quantified in Figure 5R.

In order to further address whether Slit-2, Sema 3B, and Sema 3F can affect commissural axon growth prior to crossing the floor plate, we used the so-called "turning assay" in which tissues or factors are placed to the side of explants of E11 rat dorsal spinal cord and are able to cause pre-crossing commissural axons within the explant to turn toward the exogenous tissue or source (as shown for the chemoattractant effect of floor plate tissue and COS cells secreting netrin-1: Tessier-Lavigne et al., 1988; Placzek et al., 1990; Kennedy et al., 1994) or away from the exogenous tissue or source (as shown for repellent actions of roof plate tissue and COS cells secreting BMP7: Augsburger et al., 1999). We found that COS cells secreting Slit-2, Sema3B, or Sema3F had no effect on commissural axon growth within dorsal spinal cord explants (Figures 6D–6F; n = 8, 8, and 20, respectively) under conditions where roof plate tissue repelled these axons (Figure 6A), and both

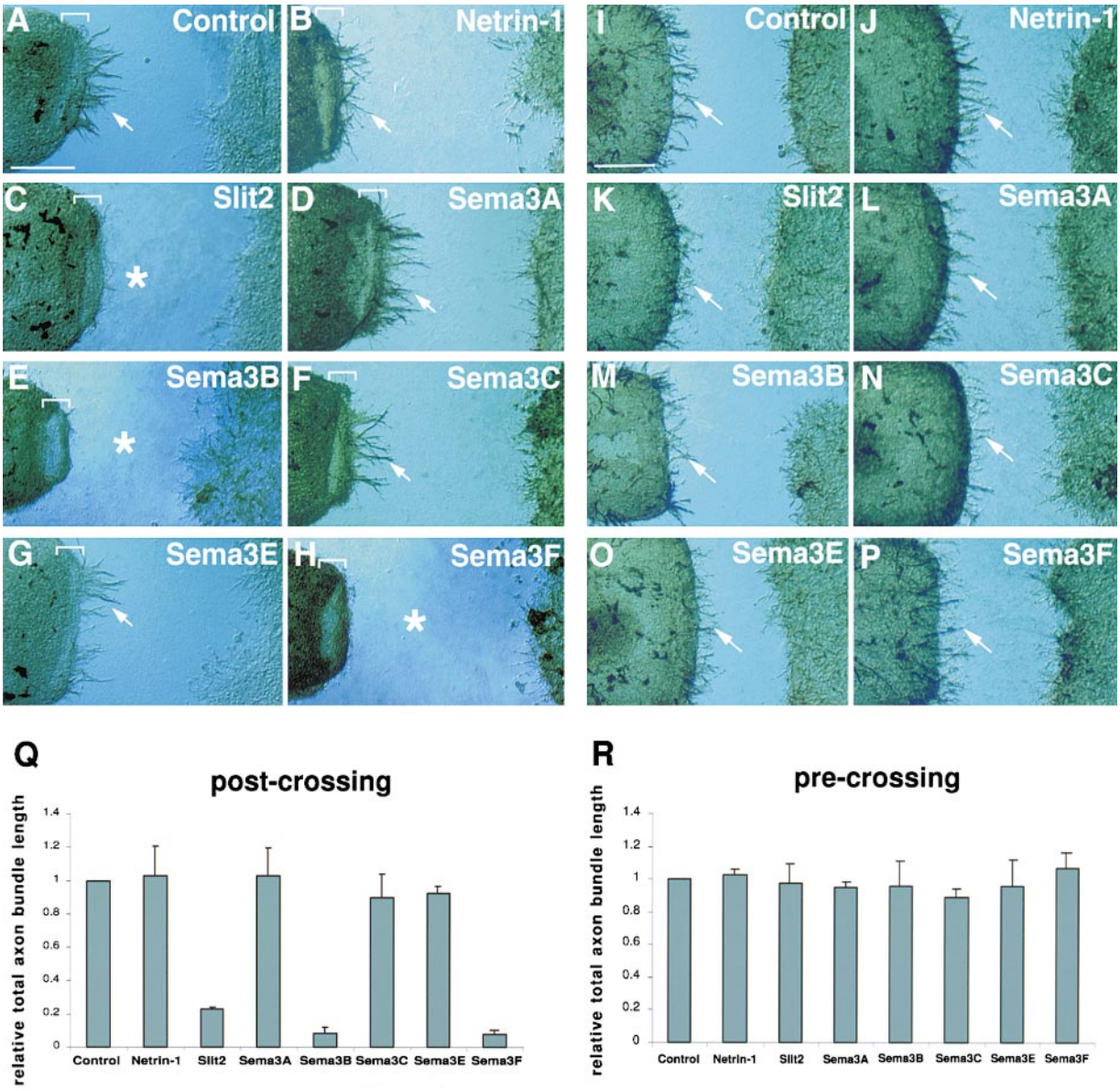


Figure 5. Slit-2 and a Subset of Class 3 Semaphorins Inhibit Post-Crossing Axons but Not Pre-Crossing Commissural Axons

(A–H) "Spinal cord plus floor plate" explants (left side of each panel) cultured with aggregates of control COS cells (A) or COS cells expressing the indicated factors (netrin-1, Slit-2, or various class 3 Semaphorins) (right side of each panel). Only Slit-2, Sema3B, and Sema3F inhibit the outgrowth of crossed axons. White arrows indicate post-crossing axons that emerge from the explants, whereas asterisks indicate the absence of the post-crossing axons in the presence of Slit-2, Sema3B, or Sema3F.

(I–P) Dorsal spinal cord explants grown with netrin-1 to elicit outgrowth of uncrossed commissural axons were cultured with aggregates of control COS cells (A) or COS cells expressing the indicated factors (netrin-1, Slit-2, or various class 3 Semaphorins). None of these factors inhibits the outgrowth of pre-crossing axons (indicated by white arrows).

(Q and R) Quantification of the inhibitory effect of the different factors on post-crossing axons (Q) and pre-crossing axons (R).

Scale bar: 200 μm.

floor plate tissue and COS cells secreting netrin-1 attracted these axons (Figures 6B and 6C). As a positive control for activity, other Slit-2-, Sema3B-, and Sema3F-secreting COS cell aggregates in these experiments were found to have repulsive or inhibitory activity on sympathetic axons and post-crossing commissural axons (data not shown). In separate experiments, we performed similar "turning assays" using not pieces of dorsal spinal cord but rather explants of the entire intact spinal cord (including the floor plate), which were cultured as "closed books" with COS cell aggregates placed alongside. In these "entire spinal cord" explants, commissural axons normally project all the way to the floor plate, and just as in dorsal explants, they were attracted by cells secreting netrin-1 but did not show any responses to cells secreting Slit-2, Sema3B, or Sema3F (data not shown). Thus, commissural axons are not repelled by these factors even as they approach the floor plate; they apparently become responsive to the repellents only upon crossing.

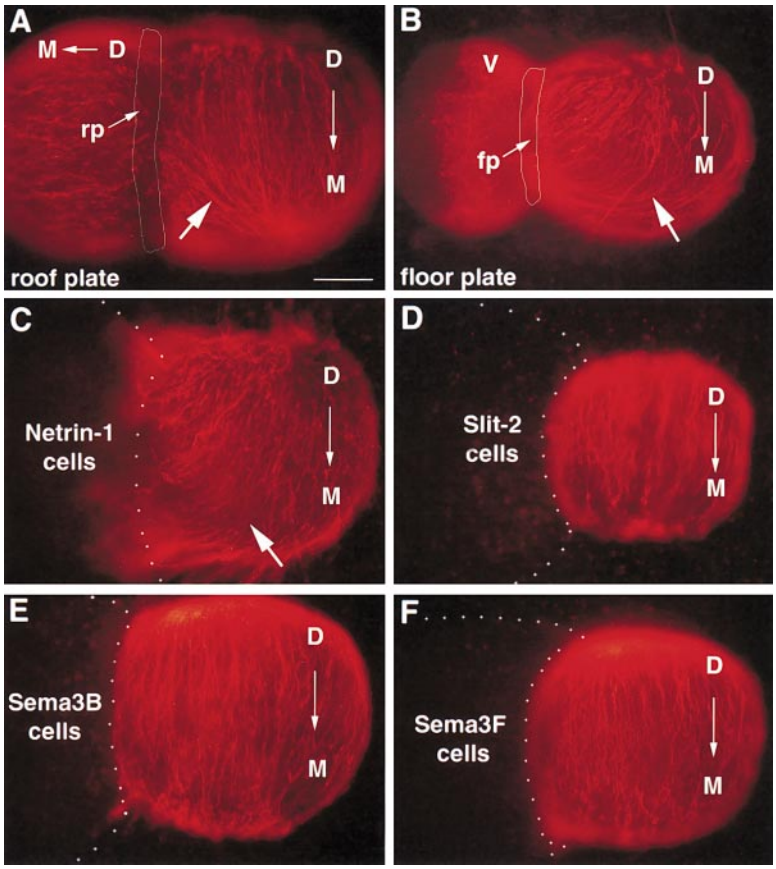


Figure 6. Slit-2, Sema 3B, and Sema 3F Do Not Repel Pre-Crossing Commissural Axons in the Dorsal Spinal Cord

In all panels, an E11 rat dorsal spinal cord explant, oriented dorsal (D) up and medial portion (M) down, was cultured in the presence of various tissues or COS cell aggregates placed on the left side of the explant. After culturing for 40 hr, whole-mount TAG-1 immunohistochemistry was performed on the explants to visualize pre-crossing commissural axons.

(A) Roof plate (rp) tissue from a piece of E11.5 mouse dorsal spinal cord (dorso [D]–medial [M] orientation is horizontal) repels commissural axons within the rat dorsal spinal cord explant (white arrow).

(B and C) Commissual axons are attracted (white arrow) by a piece of E11.5 mouse floor plate (fp) tissue (attached to ventral spinal cord [V]) (A) or by COS cells secreting netrin-1 (C).

(D–F) COS cells secreting Slit-2 (D), Sema3B (E), or Sema3F (F) neither attract nor repel commissural axons.

Scale bar: 100 μm.

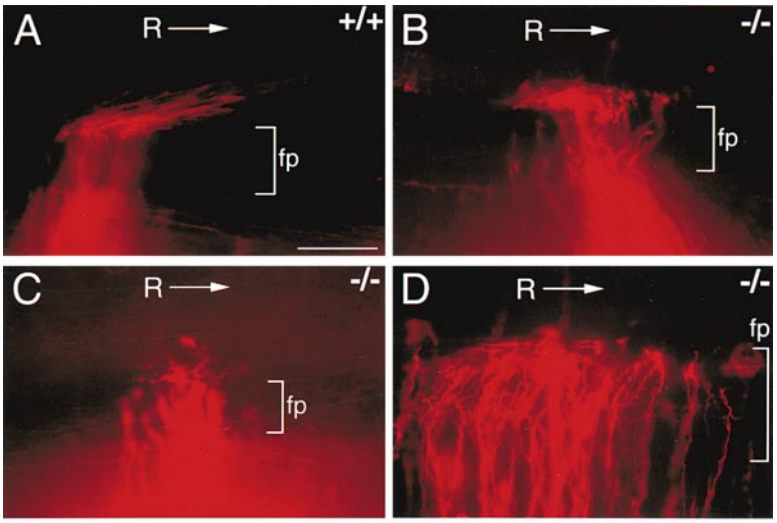
Neuropilin-2 Is Required for Normal Commissural Axon Pathfinding during and after Midline Crossing

Since the inhibitory effects of Sema3B and 3F are expected to be mediated by a neuropilin-2-dependent mechanism, we examined whether there were any defects in the projections of commissural axons at the midline in a neuropilin-2 knockout mouse that we have previously studied (Chen et al., 2000). The neuropilin-2 allele in this mouse is a severe hypomorphic allele or near null (Chen et al., 2000). No defects in commissural axon trajectories were reported during the period of initial growth of commissural axons to the floor plate (i.e., prior to E11.5–E12.5) in this knockout mouse (Chen et al., 2000) or in an independently derived neuropilin-2 knockout mouse (Giger et al., 2000).

In contrast to the absence of defects before floor plate crossing, clear defects in pathfinding at the midline were observed in homozygous mutant neuropilin-2 embryos at E11.5 and E12.5 (Figure 7). Figure 7A shows the projections of commissural axons in a wild-type E11.5 embryo visualized in an open book preparation, with commissural axons labeled by injection with DiI in the dorsal spinal cord. As shown in previous studies (Bovolenta and Dodd, 1990), commissural axons cross the floor plate in a well-organized fashion and turn sharply rostrally in wild-type embryos at these stages (Figure 7A; rostral is to the right in all panels in this figure). In homozygous mutant embryos at E11.5, several highly penetrant phenotypes were observed. In many cases, several types of defects could be observed simultaneously in a given cohort of axons labeled with a single DiI injection, as illustrated in Figure 7B. As shown, many axons appeared disorganized in the floor plate while crossing. The punctate, club-like appearance of DiI at the end of some axons within the floor plate suggests that some growth cones may have stalled while crossing. Many axons inside the floor plate appeared to be less straight and more "wavy" than in controls. Finally, many axons that did cross the floor plate made mistakes in the direction of their turn so that axonal trajectories were randomized along the anterior–posterior axis. Two other examples of the types of defects that were observed are shown in Figures 7C and 7D. Both show additional examples of wavy and spiraling axons, and axons in Figure 7C also appear to wander on the contralateral side after crossing.

The type of defects that were observed in the mutants could be placed in four categories as shown in Figure 7E. Defects were observed only within the floor plate ("spirals/zigzags" and "stalling") and after floor plate crossing ("anterior–posterior polarity errors" and "wandering") (Figure 7E); no defects were observed before floor plate crossing. Within a given cohort of axons labeled by a single DiI injection, we usually observed multiple types of defects. Thus, in E11.5 homozygous mutants, some wavy and spiraling axons were observed in about forty percent of the injections; some stalling axons were seen in over a third of the injections; some anterior–posterior projection errors were seen in over a third of the injections; and the most common error was overshooting and wandering of axons after crossing (seen in almost two-thirds of the injection sites) (data not shown).

Because of the presence of multiple types of projection defects that were present to varying extents in any given cohort of neurons, we decided to simplify the quantification of the extent of defects by classifying the appearance of the behavior of the entire group of axons



E

Types of Defects Observed	Inside the Floor Plate		After Crossing	
	Spirals/Zigzags	Stalling	Anterior-posterior Polarity errors	Wandering
Diagrams of Abnormal Projections				

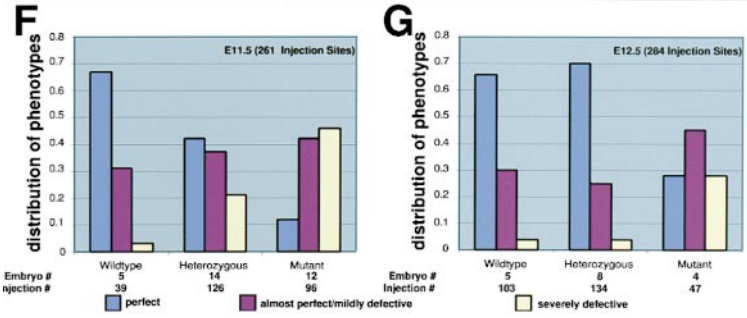


Figure 7. Neuropilin-2 Is Required for Normal Midline Commissural Axon Pathfinding In Vivo

(A–D) Visualization of commissural axon behavior at the floor plate (fp) in a wild-type E11.5 mouse embryo (A) and in three homozygous mutant neuropilin-2 E11.5 mouse embryos (B–D). Commissural axons are visualized following DiI injection in the dorsal spinal cord (off the bottom in each panel) in the "open book" configuration. Rostral (R) is to the right in each panel (indicated by arrow). In wild-type (A), commissural axons cross and turn rostrally in a very stereotyped fashion. A first example of pathfinding in a mutant embryo (B) shows randomization of the anterior–posterior projection patterns of commissural axons after exiting the floor plate, wavy axons and stalling growth cones inside the floor plate (note that the "waviness" starts approximately at the floor plate). A second example (C) shows commissural axons that are overshooting and wandering into the contralateral ventral spinal cord region after floor plate crossing (note that the full extent of wandering is not captured by this picture in a single focal plane; wandering was actually seen in multiple focal planes in most cases). A third example (D) shows spiraling and wavy trajectories inside the floor plate (note again that the waviness is seen inside the floor plate, not before the floor plate). Scale bar: (A)–(C), 100 μm; (D), 66.7 μm.

(E) Summary of commissural misrouting phenotypes in neuropilin-2 mutant mice.

(F and G) Histograms documenting abnormalities in commissural axon crossing in E11.5 (F) and E12.5 (G) mouse embryos. For each injection of DiI into a wild-type, heterozygous, or homozygous mutant embryo, the behavior of the cohort of labeled commissural axons at the floor plate was classified as "perfect" (blue bars), "almost perfect/mildly defective" (purple bars), or "very defective" (yellow bars). The numbers of embryos studied for each genotype and age, and the number of DiI injection sites in all these embryos, are listed in each case.

labeled in any given injection as "perfect," "almost perfect/mildly defective," or "severely defective." Figures 7F and 7G show histograms of the distribution of phenotypes seen with a large number of injections in wild-type, heterozygous, and homozygous mutant embryos at both E11.5 (Figure 7F) and E12.5 (Figure 7G); in these experiments, the phenotypes were scored blind (without knowledge of the genotype of the embryos). Using these categories, in wild-type embryos at both E11.5 and E12.5, about 66% of the axon cohorts showed "perfect" behavior (as in Figure 7A), about 30% showed "almost perfect/mildly defective" behavior (in which just a few axons had abnormal appearances), and less than 4% were "severely defective" (i.e., large numbers of axons showed defects). The highest penetrance of severe defects was observed in E11.5 homozygous mutant embryos, in which close to 50% of axon cohorts were classified as "severely defective" and only about 10% as "perfect." Interestingly, the frequency of "severe defects" was also higher in heterozygous E11.5 embryos than in wild-type embryos, although it was not as great as in homozygous embryos. At E12.5, in contrast, the distribution of phenotypes in wild-type and heterozygous embryos were indistinguishable, and the frequency of "severe defects" was lower in homozygous embryos than at E11.5. Taken together, these results demonstrate an essential role for neuropilin-2 in commissural axon pathfinding at the ventral midline in vivo.

Discussion

The development of an assay in which spinal commissural axons are first made to cross the floor plate before being confronted with tissues or guidance cues has enabled us to dissect the changes in responsiveness of these axons during midline crossing. Paralleling previous studies in *Drosophila*, we show that commissural axons acquire responsiveness to a midline repellent activity upon crossing the midline and that Slit proteins may contribute to this activity. We extend those observations, however, by showing that ventral spinal cord

tissue also secretes a repellent activity—perhaps involving Slit-2—to which the axons become responsive upon midline crossing, providing an explanation for why the axons do not reenter the ventral spinal cord. While Slit proteins may contribute to the repellent activities in both floor plate and ventral spinal cord, our study also implicates the class 3 Semaphorins Sema3B and Sema3F, which are high-affinity ligands for neuropilin-2 receptors on commissural axons, in mediating the repellent actions of floor plate and ventral spinal cord. The finding of projection defects in a neuropilin-2 knockout mouse supports this hypothesis. Taken together, our results suggest that midline recrossing in vertebrates is prevented not just by the loss of responsiveness to positive factors at the midline, but also the acquisition of responsiveness to negative factors. They also support a model in which commissural axons are forced, or squeezed, out of the gray matter of the nervous system into surrounding fiber tracts by repellents secreted by both the floor plate and the ventral spinal cord.

An Assay for Spinal Commissural Axon Behavior after Midline Crossing

In previous studies (Brose et al., 1999; Li et al., 1999), no effect of Slit proteins was observed on commissural axons. Our results here show that this failure reflected a limitation of the assays used, as commissural axons were tested for responsiveness prior to midline crossing. We confirm that Slit-2 does not prevent commissural axon outgrowth nor repel commissural axons prior to midline crossing—even as they approach the floor plate—consistent with those previous studies. Using our novel assay, however, we find that both floor plate cells and Slit-2 do function to inhibit outgrowth of post-crossing axons from spinal cord plus floor plate explants. The fact that the axons show this responsiveness after crossing but not before is consistent with the switch being triggered by axonal encounter with the floor plate.

Several lines of evidence support the contention that the axons emerging from the cut edge of the floor plate in our assay are post-crossing commissural axons. The results of both anterograde and retrograde labeling experiments using DiI are consistent with the axons being commissural axons, based on the location of labeled cell bodies. The fact that the axon segments emerging from the floor plate express L1 and DCC but not TAG-1, similar to antigen expression patterns on post-crossing commissural axons in vivo, provides further support. Thus, many, and perhaps all, of the axons that emerge from the cut edge of the explant are likely to be commissural axons. We cannot, however, completely exclude that some other axons, such as motor or association axons, are among the emerging axons, even if this is highly unlikely. It is important to note that this does not affect any of our conclusions, since the inhibitory effects of tissues and factors that we observe are essentially fully penetrant so that if there are noncommissural axons among the emerging axons, we would simply conclude that they must have the same responsiveness profile as post-crossing commissural axons. Finally, although the axons may be mostly or entirely commissural axons, it is expected that they will be a mixture of developing commissural axons and of regenerating axons that had already crossed the floor plate but were cut during preparation of the explants. Again, the fact that all the axons respond in the same way indicates that if there are both developing and regenerating commissural axons, then they all behave the same way.

It is important to contrast our assay with that of Shirasaki et al. (1998), which also evaluated responses of post-crossing axons to secreted factors. Their assay, however, used explants of hindbrain, where the axons that cross the midline do not immediately turn to grow alongside the midline but rather continue on the same trajectory, projecting into the contralateral ventral hindbrain gray matter. The fact that the axons continue growing straight after crossing is what made it possible in those experiments to ask whether tissues or cells placed to the side of the post-crossing axons could deflect them from this straight trajectory within the hindbrain tissue (rather than within a collagen matrix) and to show that the axons lose responsiveness to the attractive effects of floor plate and netrin-1 upon crossing. By the same token, however, the experiments also showed that the axons do not acquire a repulsive response to floor plate cells (indeed, the axons showed no responses to the floor plate whatsoever), superficially suggesting a major difference with the results in spinal cord and in *Drosophila*. We would interpret the straight trajectory of these axons as showing that hindbrain commissural axons do not acquire responsiveness to a repellent in ventral hindbrain immediately upon crossing (although, since they eventually do turn, it is possible that the acquisition of the responsiveness is simply delayed). Thus, the specific feature of hindbrain commissural axons (their continued straight growth after crossing) that made them useful for testing responses to floor plate cells appears to make them unsuitable for studying acquisition of responses to repulsive activities. It is tempting to speculate that acquisition of responses to repulsive factors may only occur at the site where the axons subsequently turn to grow parallel to the midline. Put another way, for these hindbrain axons, it remains possible that what counts as the "extended midline" is the entire region in which their post-crossing axons continue to grow straight and prior to turning. If so, then the apparent difference between these axons and *Drosophila* and vertebrate spinal axons might indeed only be superficial, as the hindbrain axons might acquire repulsive responses when they reach the edge of the "extended midline." This could be tested by developing an assay similar to ours but using "hindbrain plus extended midline" explants.

We had set out to develop this novel assay because of our interest in commissural axons in the spinal cord. In contrast to the axons in the hindbrain, spinal commissural axons both turn immediately and also exit the gray matter after crossing, projecting in adjacent fiber tracts. Since the axons hug the floor plate after crossing, it was not possible to use an assay like that of Shirasaki et al. (1998) to ask whether floor plate can deflect the axons. This led us to the novel experimental design, in which we examined the behavior of the axons in a collagen gel immediately after they have crossed the floor plate. Our experimental design also involves asking whether the tissues or cells can prevent the outgrowth of post-crossing axons into the collagen that, strictly speaking, assesses inhibitory activities rather than repulsive activities. We could not ask whether tissues or cells placed to one side of the emerging axons caused a deflection of the axons away from the source because of the well-documented fact that highly fasciculated axons growing in collagen gels (like those examined here) are not easily

deflected from their trajectory (e.g., Tessier-Lavigne et al., 1988; Richards et al., 1997). We have nonetheless referred to the activities we observed as "repulsive" because it is thought that many (or most) factors that are inhibitory in some assays can be repulsive in others (and vice-versa) and because the factors we pinpointed—Slit and Semaphorin proteins—are well known to be repulsive in other contexts.

Both Slit and Semaphorin Proteins Are Implicated in Post-Crossing Axon Repulsion

A pleasing result from this study is that, as in *Drosophila*, spinal commissural axons acquire responsiveness to at least one Slit protein (and perhaps all three) upon midline crossing. Whether this involves upregulation of vertebrate Robo receptor expression on the commissural axons after midline crossing remains to be determined. A surprising aspect of our results, however, was the finding that the class 3 Semaphorin Sema3B likely contributes to the repulsive floor plate activity as well, since its mRNA is expressed by floor plate cells and it repels post-crossing axons in our assay. This repulsive action is likely mediated by the high-affinity Sema3B receptor neuropilin-2, which is expressed by these axons. (Neuropilin-2 is likely only the ligand binding portion of the Sema3B receptor, with signaling presumably mediated by a plexin family member such as plexin-A3, which is expressed by these neurons [Takahashi et al., 1998; Tamagnone et al., 1999].) Thus, in contrast to *Drosophila*, where a single Slit protein is thought to account for all the midline repulsive activity, in vertebrates the task of repulsion of post-crossing axons by midline cells appears to be shared by at least three Slit proteins and one Semaphorin.

Our studies also revealed for the first time in any organism that crossing axons also acquire responsiveness to a repellent activity from the ventral portion of the nervous system. This is the terrain that the axons have traversed immediately before reaching the midline and which was therefore permissive for growth prior to crossing; after crossing, however, it becomes repulsive to the axons. This repulsive activity again appears to involve both Slit and Semaphorin proteins, since Slit-2 is expressed in the motor column (Brose et al., 1999; Li et al., 1999) and since Sema3F (another high-affinity neuropilin-2 ligand) is expressed throughout the mantle layer of the entire spinal cord (including the ventral spinal cord but excluding the floor plate). The existence of this repulsive activity should help prevent the axons from reentering the ventral portions of the nervous system. In fact, the repellent actions of the floor plate and the ventral spinal cord together should help squeeze the commissural axons out of the gray matter of the spinal cord entirely after they have crossed the midline. If Slit-2 and/or Sema3F proteins are also displayed on motor axons, then they might also help organize post-crossing commissural axons within the regions of the fiber tracts that motor axons traverse, a possibility suggested for Slit-2 (Li et al., 1999).

The analysis of a neuropilin-2 knockout mouse supports the involvement of the class 3 Semaphorins in regulating midline crossing of commissural axons. A frequent defect observed in the mutants is the apparent stalling out of the axons in the floor plate, which is consistent with the existence of insufficient inhibitory activity within the floor plate to help push the axons out of the midline region once they have started crossing it. Interestingly, in these cases of stalling, many or all the axons stall out at the contralateral floor plate edge; this is reminiscent of the situation in *robo* mutants in *Drosophila*, where the axons can recross the midline but do not stall out in the middle, apparently because of the operation of a weaker repulsive mechanism (also involving Slit but mediated by some other receptor, perhaps Robo-2 [Kidd et al., 1999]). The presence of residual inhibition at the midline in the neuropilin-2 knockout mice might similarly explain why axons grow to the contralateral edge of the floor plate.

In addition, the defects are only partially penetrant and also seem to be corrected as the embryo matures, indicating the operation of redundant guidance mechanisms. These mechanisms presumably include the Slit proteins but also possibly other nondiffusible guidance cues, such as ephrinB2, which a recent descriptive analysis has suggested might be involved in regulating midline guidance as well (Imondi et al., 2000). EphrinB2 might, in fact, be a good candidate for the short-range repellent activity of floor plate cells documented in chick (Stoeckli and Landmesser, 1995; Stoeckli et al., 1997), to which commissural axons appear to be sensitive even prior to crossing (at least in chick).

Finally, a frequent defect seen in the neuropilin-2 knockout mouse is in the direction of turns after crossing. In wild-type embryos, commissural axons turn rostrally with a high degree of precision, but in the mutants, the axons often make errors, turning caudally. It is not clear whether these defects reflect a primary role for neuropilin-2 in interpreting axon guidance information along the anterior–posterior axis or whether they are simply a secondary consequence of axon stalling in the floor plate.

Entering and Leaving Fiber Tracts: A Global Hypothesis

The exiting of spinal commissural axons into the ventral funiculus from the gray matter after midline crossing is representative of the behavior of large numbers of other axons up and down the neuraxis, which grow to their targets by coursing through the gray matter to some exit point where they join and grow in fiber tracts, only later leaving the tracts to reenter the gray matter and to connect with their target cells.

We suggest that the mechanism we have described here may be representative of those operating throughout the nervous system to propel axons out of the gray matter into fiber tracts. It may be true quite generally that as axons leave the gray matter, they acquire responsiveness to both midline and gray matter repellent activities. It is intriguing in this regard that Sema3F and Sema3B, between them, are expressed throughout much of the gray matter and midline. In fact, the finding that Sema3F is expressed throughout the mantle layer, essentially everywhere where axons grow within the spinal cord (and in other brain regions as well), is hard to square with a role in guidance *within* the mantle layer. Rather, it seems more likely that it functions to prevent axons from entering or reentering the mantle layer and thus helps keep them in fiber tracts. The Slit proteins may also play such a role quite generally, since their mRNAs, after initially being most highly expressed in midline tissues, later become more widely expressed throughout the gray matter (Brose et al., 1999; Li et al., 1999; Wang et al., 1999).

After axons have grown in fiber tracts, what permits them to reenter the gray matter? This is an issue we studied recently in the context of sensory axon collateral ingrowth into the spinal cord. Remarkably, that study implicated Slit proteins as positive regulators of sensory axon branching and ingrowth into the spinal cord gray matter (Wang et al., 1999). We proposed at that time that Slit proteins might function generally to permit axon ingrowth into gray matter from adjacent fiber tracts (see Discussion in Wang et al., 1999). Putting together these two suggestions, a global hypothesis suggests itself: axons that leave the gray matter are kept out because they acquire responsiveness to a repellent activity made by gray matter that involves Slit proteins (and Semaphorin proteins), and when they later branch back into the gray matter, they may do so because they acquire responsiveness to an attractive or permissive activity made by gray matter that may also involve Slit proteins (and perhaps also Semaphorin proteins?). Thus, in the most extreme version of this hypothesis, the axons may initially be able to grow through the gray matter because they are impervious to Slit and Semaphorin proteins and then acquire repulsive responses to these factors as they leave the gray matter, only reentering the gray matter when their responses to Slit and Semaphorin proteins switch from being repulsive to attractive. The ability of growth cones to rapidly switch their responsiveness between repulsion and attraction has been demonstrated for several types of cues, including Semaphorins, in tissue culture experiments using *Xenopus* neurons (Ming et al., 1997; Song et al., 1998; Hopker et al., 1999). Future experiments will test whether the initial exit and subsequent reentry of the gray matter is controlled by such a neatly choreographed series of changes in growth cone responsiveness—from no response, to repulsion, to attraction—to guidance cues of the Slit and Semaphorin families and help elucidate what other mechanisms are at play in regulating gray matter entry and exit.

Experimental Procedures

Collagen Gel Assays

To study the behavior of axons after floor plate crossing, E13 rat spinal cords were dissected in L15 medium into the "open book" configuration (Figure 1). A strip of spinal cord tissue (300 μm in width) was dissected out as indicated in Figure 1B with the floor plate attached. Explants were cultured in a three-dimensional collagen gel matrix as described (Tessier-Lavigne et al., 1988). The behavior of commissural axons prior to crossing was examined by dissecting out a stripe of dorsal spinal cord (300 μm in width; Figure 1B) and culturing in a collagen gel in the presence of 30 ng/ml netrin-1. Turning assays were performed as previously described (Tessier-Lavigne et al., 1988; Placzek et al., 1990; Kennedy et al., 1994; Augsburger et al., 1999).

For quantification (Figure 3), the total length of axon bundles emerging from the cut edge of explants was measured; the total axon bundle length seen in explants cultured with ventral spinal cord or floor plate was measured relative to that seen from explants cultured alone. The average relative outgrowth in each experiment was measured from four explants in each condition. For post-crossing axons, the mean of three or six experiments (for ventral spinal cord and floor plate, respectively) was calculated; for pre-crossing axons, the mean of two experiments was calculated. Similarly, the data in Figure 4 were quantified (Figures 4Q and 4R) by measuring the total axon length on the side facing the COS cells of each explant and taking the ratio of the total length of the axons of the explants exposed to one of the above guidance molecules to the total axon length of the explants exposed to control COS cells transfected with the empty expression vector. The average relative outgrowth in each experiment was measured from four explants in each condition. The mean of three experiments was calculated.

Immunofluorescence

Whole-mount immunofluorescence staining was performed as previously described (Kennedy et al., 1994 ; Colamarino and Tessier-Lavigne, 1995). Monoclonal antibodies E7 against β-tubulin (Figures 1D and 1E) and 4D7 against TAG-1 (Figure 1F) were obtained from the Developmental Studies Hybridoma Bank. The antiserum against the mouse L1 (Figure 2C) was generously provided by Dr. Carl Lagenaur at the University of Pittsburgh. The anti-DCC antibody AF5 was from CalBiochem.

In Situ Hybridization

In situ hybridization was performed as described (Frohman et al., 1990) using cryosections of 10 μm thickness. The mouse Sema3A probe was prepared by in vitro transcription from a cDNA fragment (nt 429–1610, GenBank X859930). The mouse Sema3B probe was a kind gift from Dr. S. Strittermatter, Yale University. The Sema3C probe was derived from the expression vector for a Sema3C-AP fusion protein (Chen et al., 1997). The Sema3E probe was a kind gift from Dr. C. Christensen, Institute of Cancer Biology, Copenhagen. The mouse Sema3F probe was generated from a cDNA fragment (nt 200–1140, GenBank AF080090).

cDNA Expression Constructs

The netrin-1, Sema3A, Sema3F, and Slit-2 expression vectors were as described (Serafini et al., 1994; Messersmith et al., 1995; Chen et al., 1998; Brose et al., 1999). The Sema3B expression construct was a kind gift of Dr. S. Strittmatter. The Sema3C expression vector was as described in Chen et al. (1998). The Sema3E cDNA in pBluescript SK was a kind gift from Dr. C. Christensen; the full-length Sema3E coding region was subcloned into pcDNA3 for COS cell expression.

DiI Tracing

Spinal cords of E11.5 neuropilin-2 mutant and wild-types embryos were prepared in an open book configuration, fixed with 4% paraformaldehyde, and injected with DiI (Molecular Probes) into the dorsal region. DiI was allowed to diffuse to label commissural axons, enabling their visualization by fluorescence microscopy.

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